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Ectopic expression of rice *Xa21* overcomes developmentally controlled resistance to *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT

Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) activates the innate immune response. The rice PRR, *XA21*, confers robust resistance at adult stages to most strains of the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Seedlings are still easily infected by *Xoo*, causing severe yield losses. Here we report that *Xa21* is induced by *Xoo* infection and that ectopic expression of *Xa21* confers resistance at three leaf stage (three-week old), overcoming the developmental limitation of *XA21*-mediated resistance. Ectopic expression of *Xa21* also up-regulates a larger set of defence-related genes as compared to *Xa21* driven by the native promoter. These results indicate that altered regulation of *Xa21* expression is useful for developing enhanced resistance to *Xoo* at multiple developmental stages.

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1. Introduction

At least 10% of global food production is lost to plant disease [1]. Resistance to many of these diseases is often developmentally controlled such that only adult plants are resistant [2]. For example, development-controlled disease resistance is observed in wheat/*Puccinia recondita* [3], Maize/*Puccinia sorghi* [4], tomato/*Cladosporium fulvum* [5], and *Arabidopsis*/*Pseudomonas syringae* interactions [6]. Despite the economic importance of seedling resistance, little is known about the biochemical and molecular mechanisms involved in this regulation.

Plant innate immune systems rely on monitoring the presence of pathogen through PRRs (which recognize PAMPs) and nucleotide-binding site leucine-rich repeat (NB-LRR) type proteins (which recognize pathogen effectors) [7–9]. The rice PRR, *XA21*, recognizes the PAMP *Ax21* (Activator of *XA21*-mediated immunity), present in all *Xanthomonas* and *Xylella* species [10–12]. *XA21*-mediated resistance has been shown to be under developmental control with incomplete resistance in early stages that gradually increases with development [13]. *XA3/XA26*-mediated resistance is also developmentally regulated [14]. The developmentally controlled resistance

is hypothesized to be due to transcript levels of *Xa21* and *Xa3/Xa26* [2,14]. Their expressions are low at the two-leaf stage, and then gradually increase with development. The highest level of expression is reached at the maximum-tillering stage, consistent with an increased resistance at this stage. Based on the expression result of *Xa21*, we hypothesized that ectopic expression of *Xa21* may overcome the developmental control of *XA21*-mediated resistance. Ectopic expression experiments are specifically designed to increase the abundance of the desired transcript through introduction of a transgene into the host. This strategy was applied to the study of several NB-LRR and PRR proteins. In the case of *Arabidopsis RPS2*, tomato *Pto*, and rice *Xa3*, overexpression leads to constitutive activation of downstream defence responses even in the absence of the corresponding effector or PAMPs [14–17].

Here we show that transgenic plants overexpressing *Xa21* display significantly increased resistance at the seedling stage. These results support the hypothesis that *Xa21* transcript levels are rate limiting in early stages of development.

2. Materials and methods

2.1. Plant material and growth conditions

Rice (*Oryza sativa* L.) plants [cultivar Kitaake (Kit)] were maintained in the greenhouse. The growth chamber was set on a 16 h light and 8 h dark photoperiod, 28/26 °C temperature cycle, and 85/90% humidity. Healthy and well-expanded leaves from three-

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or six-week-old rice plants, Kit and transformed Kit were used for *Xoo* PXO99Az inoculation and nucleic acid or protein extraction.

2.2. Construction of the *Ubi Myc-Xa21*, *Ubi Xa21-CFP*, *Nat Myc-Xa21* and *Nat Xa21-CFP* plasmids for expression in rice

To construct the *Ubi Myc-Xa21* plasmid, a 5' fragment of *Myc-Xa21* was PCR-amplified using primers, 5'-AAAGGATCC-AACATCTCTCGTGTCTT-3'/5'-GGCTGAGCTCCGGTGGTAT-3' and template *pC822-cMyc-Xa21* [18,19]. This 420 bp 5'-fragment was cut with BamHI/SacI at the ends and cloned, together with a 4.2 kb SacI/SpeI *Xa21* 3'-fragment, into the pBluescript II SK-vector to create a promoterless full-length *Myc-Xa21* gene. The 5' end of this gene was confirmed by sequencing. This *Myc-Xa21* gene was excised with BamHI/SpeI and subcloned into the Ubi-CAMBIA-1300 vector using the same enzyme sites to generate plasmid Ubi *Myc-Xa21*/C1300.

To fuse the XA21 protein to the cyan fluorescent protein (CFP), a 380 bp 3' fragment of the *Xa21* gene was PCR-amplified using primers, 5'-TGCATCAACGCATGGAGATA-3'/5'-AATTCCATGGGAAATTCAGGCTCCACCTT-3'. This fragment removed the stop codon and the EcoRI site located immediately in front of the stop codon. EcoRI was used to digest the 5' region and NcoI to digest the 3' region of *Xa21* 3' PCR product. Meanwhile, the CFP gene was excised from the pECFP plasmid (Clontech) using NcoI/SpeI. These two fragments were joined at the NcoI site and cloned into pBluescript II SK-, predigested with EcoRI/SpeI, to create plasmid *Xa21* 3'-CFP/SK. The *Xa21* portion and the junction were confirmed by sequencing. The *Xa21* 3'-CFP fragment was excised with EcoRI (the second EcoRI site, coming from the pECFP plasmid, is located downstream next to the end of the CFP gene) and used to replace the 380 bp EcoRI fragment in the original promoterless (as described above) *Xa21* gene, creating *Xa21-CFP*. To create the Ubi XA21-CFP/C1300 construct, the *Xa21-CFP* fragment was excised with BamHI/SpeI and cloned into the Ubi-CAMBIA-1300 vector predigested with BamHI/SpeI as described above. The Nat XA21-CFP/C4300 construct was generated by ligating the SacI/SpeI *Xa21-CFP* 3' fragment and the KpnI/SacI *Xa21* 5' fragment jointly with CAMBIA-4300 pre-digested with KpnI/XbaI. The Nat *Myc-Xa21*/C1300 construct was previously described [19].

2.3. Expression analysis

For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, total RNAs were extracted from leaves using TRIzol[®] reagent (Invitrogen) after each treatment. Then the RT reaction was performed following the manual for QuantumRNA 18S Internal Standards (Ambion). PCR analyses were performed with primers pairs, 5'-TCATCACTCTACTTGCTTATA-3'/5'-GAATTCAAGGCTCCACCTTC-3' (for *Xa21_cds*), 5'-GTTTTATTGCCACTTCAGA-3'/5'-GGAAGCCCC-TCCACCTCCCTCATC-3' (for *Xa21_utr*). After 28 cycles, the amplified products were resolved by gel electrophoresis.

For qRT-PCR, the total RNA was treated with RNase free DNase (Promega), purified using Macherey-Nagel Nucleospin RNA II kit and quantified using ND-1000 spectrophotometer (Nanodrop). cDNA was synthesized from 10 µg of total RNA in 40 µl volume using M-MLV RT (Invitrogen) followed by RNase treatment (NEB). The cDNA was cleaned using Zymo DNA clean and concentrator kit and eluted in 100 µl of 1 mM Tris-HCl. The cDNA was diluted to 20 folds and 4 µl of it was used for each reaction. Gene-specific primers were designed using PRIMER EXPRESS version 2.0 (PE Applied Biosystems, USA) and checked for their specificity using Blast tool of National Center for Biotechnology Information (NCBI). Primer sequences have been given in Supplementary Table 1.

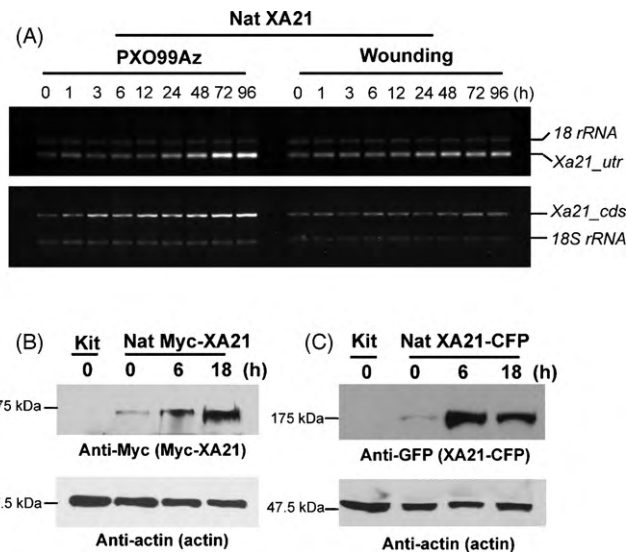


Fig. 1. *Xa21* is induced after *Xoo* strain PXO99Az inoculation. (A) Total RNA was extracted from Kitaake, mock-treated Nat XA21 (Wounding), and *Xoo* PXO99Az-inoculated Nat XA21 (*Xoo* PXO99Az) at the indicated time points. RT-PCR was performed with the specific primers designed from *Xa21* UTR (*Xa21_utr*) and *Xa21* CDS (*Xa21_cds*) regions, respectively. Control RT-PCR reactions were carried out with *18S rRNA*. (B) Total protein extracts from *Xoo* PXO99Az-inoculated Nat *Myc-XA21* (T₃, line 20-4) at the indicated time points were used for protein gel blot analysis. Equal amounts of total protein from non-transgenic (Kit) and Nat *Myc-XA21* were analyzed by SDS-PAGE, and immunoblotted with anti-Myc antibody. *Myc-XA21* gives bands at about 140 kDa. Experiments were repeated over three times with similar results. Equal loading of total proteins was confirmed by anti-actin antibody. (C) Total protein extracts from *Xoo* PXO99Az-inoculated Nat XA21-CFP (T₂, line 15A-1-7) at the indicated time points were used for protein gel blot analysis using anti-GFP antibody. XA21-CFP gives bands at about 180 kDa. Experiments were repeated over three times with similar results. Equal loading of total proteins was confirmed by anti-actin antibody.

Each QPCR reaction was performed in 10 µl of volume using Bio-Rad CFX-96[™] detection system (Bio-Rad) with Ssofast[™] EvaGreen[®] supermix (Bio-Rad) using following PCR conditions: (95 °C for 3 s and 60 °C for 3 s) 40 cycles followed by melt curve analysis. The data was normalized using actin as endogenous control and analyzed to calculate relative expression values using $\Delta\Delta C_t$ method. Three technical and three biological replicates were performed for each sample and standard error was calculated.

3. Results

3.1. *Xa21* expression is induced by *Xoo* infection

To determine if *Xa21* expression is modulated by *Xoo* strain PXO99Az expressing Ax21, its expression pattern was monitored in six-week-old transgenic rice plants carrying *Xa21* gene under the control of its native promoter (Nat XA21 23A-1-14, homozygous T₂) (Fig. 1A). For reverse transcriptase (RT)-PCR analysis, specific primer sets in 3'-untranslated region (utr) and coding sequence (cds) of *Xa21* were used. As expected, transcripts corresponding to *Xa21* in wild type Kitaake (Kit) were not detectable at any time point after *Xoo* PXO99Az inoculation, indicating that primers for *Xa21* are specific (data not shown). In contrast, Nat XA21 plant wounded by cutting the leaf tip with scissors induced *Xa21* gene to moderate level. In *Xoo*-inoculated Nat XA21, *Xa21* was induced in RT-PCR analysis, performed with both primer pairs (labeled as *Xa21_utr* and *Xa21_cds*) with maximum accumulation at 72 h after inoculation (HAI). This result suggests that Ax21 and XA21 interaction regulates XA21-mediated immune response as well as the transcription level of *Xa21*. 18S ribosomal RNA (*18S rRNA*) was used as an internal control.

To investigate if accumulation of *Xa21* transcripts correlates with the XA21 protein after *Xoo* PXO99Az inoculation, we generated transgenic Kit lines possessing an N-terminal Myc-epitope-tagged XA21 (Nat Myc-XA21) or a C-terminal CFP-tagged XA21 (Nat XA21-CFP), under the control of its native promoter. At the six-week-old adult stage (9–10 leaves), non-transgenic Kit plants are susceptible to *Xoo* PXO99Az. In contrast, the transgenic Nat Myc-XA21 (T₃, 20-1) and Nat XA21-CFP (T₁, 15A-1) plants were fully resistant to *Xoo*, indicating that the proteins are biologically equivalent to the native XA21 protein (Fig. S1A and B). We then investigated if XA21 is accumulated using anti-Myc or anti-GFP antibodies after *Xoo* PXO99Az inoculation (Fig. 1B and C). In the absence of *Xoo* (0 time point), Myc-XA21 protein of 140 kDa could be detected slightly, but not in Kit control plants (Fig. 1B). At 6 HAI, the protein started to accumulate and continuously increased till 18 HAI. We have previously reported that the XA21 protein accumulation is independent of mRNA levels after *Xoo* inoculation, because the stability of the XA21 protein is regulated through ER quality control mechanisms [20]. The anti-actin antibody has been used as an internal control to show equal loading in each lane. Anti-GFP antibody detected a 180-kDa polypeptide in transgenic plants carrying Nat XA21-CFP but not in the control line Kit (Fig. 1C). Similar accumulation pattern of XA21-CFP protein was observed after PXO99Az inoculation, indicating that XA21 protein synthesis and/or stability is increased during the defense response.

3.2. Generation of transgenic rice plants overexpressing *Xa21*

Based on the results of *Xoo* PXO99Az inoculation in Fig. 1, we hypothesized that accumulated *Xa21* transcripts and XA21 proteins may affect the resistance level to *Xoo*. To evaluate the hypothesis, we transferred *Xa21* driven by a strong constitutive promoter, maize *ubiquitin* gene promoter (*Ubi*) into Kitaake wild type and generated 21 independent transgenic lines carrying *Myc-Xa21* under the control of the *Ubi* promoter (Ubi Myc-XA21). We then analyzed the transgenic lines (T₀ generation) at six weeks of age for alterations in resistance to *Xoo* PXO99Az. Fourteen Ubi Myc-XA21 lines displayed similar level of enhanced resistance to *Xoo*. Seven lines showed no difference compared to Kitaake (data not shown). We generated T₁ progeny from each of the T₀ lines displaying enhanced resistance and analyzed the T₁ progeny for Mendelian segregation of the transgene with the enhanced resistance phenotype. 2A, 7A, 8A, and 9A of lines were chosen for further analysis. The protein levels of Myc-XA21 in six-week-old transgenic lines (T₁) displaying the enhanced resistance (2A-1, 7A-1, 8A-2, and 9A-1) were examined by Western blot analysis using anti-Myc antibody (Fig. 2A). The Ubi Myc-XA21 transgenic lines (T₁) overexpress Myc-XA21 protein compared to the Nat Myc-XA21 lines. Anti-actin antibody was used as an internal loading control. Total RNAs from Kit, homozygous Nat Myc-XA21 (20-1), and homozygous Ubi Myc-XA21 (T₃, 7A-8-101-1 and -2) were extracted. Then we performed RT-PCR with primers targeting specifically *Xa21* (Fig. 2B). Internal control, *18S rRNA*, showed constitutive expressions in all tested plant lines. Although the *Xa21* was detected in Nat Myc-XA21 to moderate level, the levels of transcripts and protein were significantly increased in Ubi Myc-XA21, demonstrating that *Xa21* under control of *Ubi* promoter was overexpressed constitutively.

3.3. Constitutive expression of *Xa21* shows enhanced resistance to *Xoo*

We then examined if the increased XA21 protein caused by its constitutive expression results in the enhanced resistance to *Xoo*. After *Xoo* PXO99Az inoculation to six-week-old plants, the lesion lengths of two independent homozygous lines (Ubi Myc-XA21 T₁, 7A-8 and 9A-12) were compared with homozygous Nat Myc-XA21

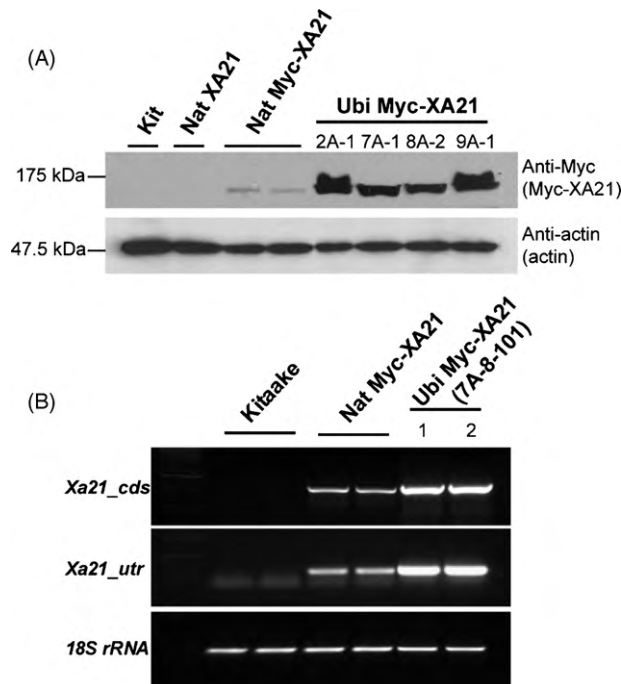


Fig. 2. Rice plants overexpressing *Myc-Xa21* under control of *Ubi* promoter (Ubi Myc-XA21) produce higher levels of XA21 protein. (A) Total proteins were extracted from six-week-old plants (Kit, Nat XA21, Nat Myc-XA21, and Ubi Myc-XA21 (T₁)) and protein gel blot analysis was performed with anti-Myc antibody. Equal loading of total proteins was confirmed by anti-actin antibody. (B) Total RNA was extracted from six-week-old Kitaake wild type (Kit), Nat Myc-XA21, or Ubi Myc-XA21 (T₃, 7A-8-101-1 and -2). RT-PCR was performed with specific primers for *Xa21* UTR (*Xa21*_utr) and *Xa21* CDS (*Xa21*_cds) regions, respectively. Control RT-PCR reactions were carried out with *18S* rRNA.

line (20-1) (Fig. S2A). At 12 days after inoculation (DAI), Kit wild type displayed susceptibility to *Xoo* PXO99Az with long lesions ranging in length over 15 cm, in contrast to the Nat Myc-XA21 line which showed 2–3 cm lesion lengths. Ubi Myc-XA21 displayed shorter lesion lengths (approximately 1 cm) compared to Nat Myc-XA21, indicating that overexpression of *Xa21* confers enhanced resistance. To quantify the effect of XA21 overexpression, homozygous Ubi Myc-XA21 (T₂, 7A-8-123), Nat Myc-XA21, and Kitaake were inoculated with *Xoo* PXO99Az and bacterial growth was monitored over time (Fig. S2B). At eight DAI, significant decrease in bacterial population was detected in the Ubi Myc-XA21 lines compared with the Nat Myc-XA21. At 12 DAI, *Xoo* strain PXO99Az populations in Ubi Myc-XA21 lines reached to 2.75×10^7 colony-forming units per leaf (cfu/leaf), which is approximately five-fold decrease compared to Nat-Myc-XA21 control (1.24×10^8 cfu/leaf).

This result was also confirmed with another Ubi XA21 lines which carry XA21 tagged with CFP (Ubi XA21-CFP). Six-week-old progenies (T₁) from self-pollinated 7B, 10B, 11B, and 18B were inoculated with *Xoo* PXO99Az. Twelve DAI, we examined for co-segregation of genotype with phenotype by PCR analysis and enhanced resistance by measurement of the length of *Xoo*-induced lesions. All segregants carrying *Ubi Xa21-CFP* displayed enhanced resistance to *Xoo* PXO99Az compared to homozygous transgenic plants carrying XA21-CFP under control of native promoter (Nat XA21-CFP 15A-1) (Fig. S3). Segregants lacking *Ubi Xa21-CFP* showed susceptibility upon *Xoo* PXO99Az inoculation.

3.4. Overexpression of *Xa21* overcomes developmentally regulated resistance

The resistance conferred by XA21 progressively increases from the susceptible juvenile two-leaf stage (approximately two-week

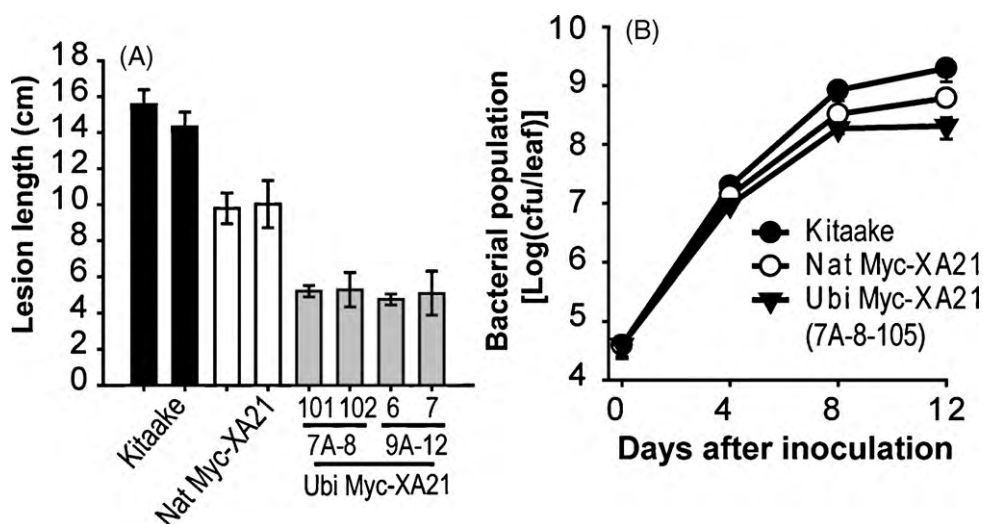


Fig. 3. Rice plants overexpressing *Myc-Xa21* (Ubi *Myc-XA21*, T₂) overcome developmental control of *Xa21*-mediated resistance to *Xoo*. (A) Lesion length development of *Xoo* PXO99Az-inoculated three leaf stage plants (three-week old), Kitaake control (Kit), Nat *Myc-XA21* and Ubi *Myc-XA21*. Each data point represents the average and standard deviation of at least four samples. (B) Plots of *Xoo* PXO99Az populations over 12 days in Kitaake control (Kit), Nat *Myc-XA21*, and Ubi *Myc-XA21* (T₂, 7A-8-105). For each time point, the bacterial populations were separately determined for three leaves. Capped, vertical bars represent standard deviation of values (cfu/leaf) from three samples. Experiments were repeated at least three times with similar results.

old) through later stages, with full resistance only at the adult stage [13]. To test if the enhanced resistance by the overexpression of *Xa21* can overcome the juvenile stage susceptibility, we inoculate the three leaf stage plants (three-week old). At this stage of development, *Xa21* rice plants are only partially resistant (approximately 40% of resistance to that of six-week-old plants) [13]. At 12 DAI, we measured the lesion length of three leaf stage plants inoculated with *Xoo* PXO99Az (Fig. 3A). The Kit control displayed susceptibility to *Xoo* PXO99Az with long lesions ranging in length from 14 to 15 cm. Homozygous Nat *Myc-XA21* (20-1) developed relatively long lesion lengths (approximately 8–10 cm), confirming that juvenile stage *Xa21* plant does not possess full-resistance observed in adult-stage *Xa21* plants. However, significantly enhanced resistance was observed in two independent homozygous lines, Ubi *Myc-XA21* (T₂, 7A-8-101, 7A-8-102, 9A-12-6, and 9A-12-7) with only 4–5 cm lesion lengths.

We quantified the effect of *Xa21* over-expression on bacterial growth by monitoring bacterial populations on homozygous Ubi *Myc-XA21* plants (T₂, 7A-8-105) inoculated with *Xoo* PXO99Az (Fig. 3B). For all growth curves until 4 days after inoculation (DAI), there was no significant difference in bacterial populations in any of the lines. However, significant difference in bacterial growth was observed at 12 DAI. Susceptible Kit plants reached approximately 1.97×10^9 colony-forming units per leaf (cfu/leaf). The bacterial populations in Nat *Myc-XA21* grew to 7.15×10^8 cfu/leaf, showing the partial resistance upon *Xoo* PXO99Az in three leaf stage. In Ubi *Myc-XA21* plants (T₂, 7A-8-105), the population leveled off at less than 2.05×10^8 cfu/leaf, indicating that developmental control of *Xa21* can be overcome by its constitutive expression.

3.5. Expression of defense-related genes correlates with *Xa21* expression levels

To elucidate the molecular mechanism to confer the enhanced resistance to juvenile and adult stages of Ubi *Xa21* transgenic lines, we examined if the constitutive expression of *Xa21* activates defense signaling pathway. Total RNAs from Kit, homozygous Nat *Myc-XA21* (20-1), and homozygous Ubi *Myc-XA21* (T₃, 7A-8-101-1 and -2) were extracted. We examined the expression of defense related genes in transgenic plants carrying *Xa21* under native or Ubi

promoter using a real-time qRT-PCR analysis in the absence of *Xoo* treatment (Fig. 4). The list of genes is given in Table S1.

The defense related gene, *PR10b*, *PBZ1*, *WRKY71*, *peroxidase*, *hsp90*, and *SDF2*, were induced in both transgenic plants. In contrast, *COI1*, a gene induced during involved in jasmonate signaling [21], *OsMT2b*, a negative regulator of oxidative burst [22], *SSI2*, implicated in cell death [23], and *OsSGT1*, a gene encoding a co-chaperone of HSP90, were up-regulated only in the Ubi *Myc-XA21* transgenic plants. These results demonstrate that overexpression of *Xa21* up-regulates a new set of defense-related genes as compared to Nat *Myc-XA21*, thus enlarging the number of possible target genes that are induced.

3.6. Enhanced resistance mediated by overexpression of *Xa21* is *Ax21*-dependent

To elucidate whether the enhanced resistance mediated by ectopic expression of *Xa21* is *Ax21*-dependent, we inoculated Ubi *Myc-XA21* with the *Xoo* PXO99 Δ *raxST*, mutant strain defective in *Ax21* biological activity [24]. At 12 DAI, Kit, Nat *Myc-XA21*, and Ubi *Myc-XA21* (T₃, 7A-8-117-1 and -2) displayed long lesions ranging in length around 12–14 cm, suggesting that *Xa21* overexpression is not able to induce resistance to *Xoo* strains lacking *Ax21* activity (Fig. 5A).

We also investigated if overexpression of *Xa21* can confer resistance to a normally virulent fungal pathogen (Fig. S5). For this experiment, we used *Magnaporthe oryzae* isolate R01-1, which is compatible with Kitaake. Ten DAI with *M. oryzae*, disease levels in Kit, Nat *Myc-XA21*, and Ubi *Myc-XA21* (T₂, 7A-8-1 and 9A-12-1) were evaluated by measuring lesion lengths. No significant difference was observed in the tested rice lines. Taken together, these results indicate that enhanced resistance by overexpression of *Xa21* still requires the presence of *Ax21*.

4. Discussion

4.1. *Xa21* expression is regulated upon *Xoo* infection

It has been previously reported that the expression of *PRR* and *NBS-LRR* genes controlling plant immunity are transcriptionally regulated under various conditions, including *Arabidopsis* *RPS2* and

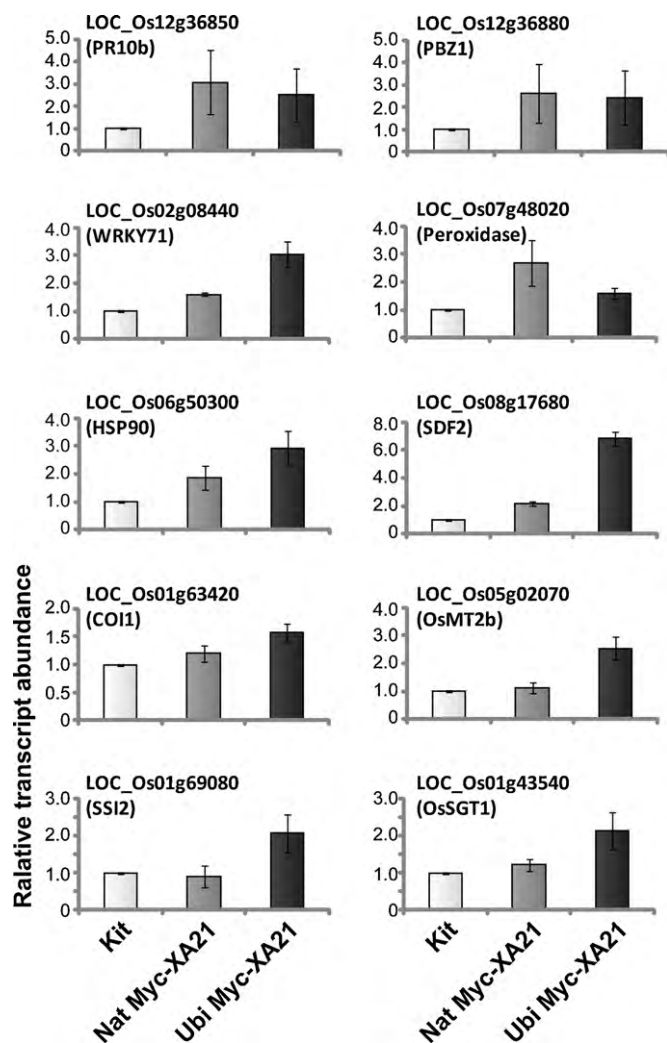


Fig. 4. Rice plants overexpressing *Myc-Xa21* (Ubi *Myc-XA21*) show increased expression of defense-related genes. Total RNA was extracted from six-week-old Kitaake wild type (Kit), Nat *Myc-XA21*, or Ubi *Myc-XA21* (T₃, 7A-8-101-1 and -2) plants. qRT-PCR was performed with specific primers for each gene. Gene expression level was normalized using actin as an internal reference.

RPM1 [25] and rice *Xa1*, *Xa27*, and *Xa3/Xa26* [14,26,27]. Although it was reported that expression of *Arabidopsis* PRR, *FLS2*, was not affected by flagellin treatment [16], a search in the gene expression database Genevestigator revealed that *FLS2* is induced by bacterial LPS, fungal chitin, and the oomycete derived NPP1 [28,29]. Those reports suggest that expression of the receptors may be regulated by their corresponding PAMPs and effector molecules.

Here we investigated if the transcript level of *Xa21* changes during response to *Xoo* treatment. We found that *Xa21* is induced in response to *Xoo* and wounding. *Arabidopsis* *FLS2* is also activated by wounding [30]. Because wounds provide entry sites for potential pathogen, when wounded, plants respond quickly by protecting them from subsequent pathogen [30,31]. Therefore, an increased availability of PRRs such as *XA21* and *FLS2* in the plasma membrane may accelerate responding to subsequent bacterial infection through the wounding tissue.

4.2. *XA21* up-regulates genes involved in pathogen-induced defense response

To explain the enhanced resistance to *Xoo* in transgenic plants overexpressing *Xa21*, we focused on the genes previously shown

to be induced during the rice defense response [32–34] and analyzed their expressions in the transgenic lines carrying *Xa21* under the control of its native or *Ubi* promoter. Our results indicate that PR and other defense-related genes are upregulated in *Xa21*-overexpressing lines compared with the Kitaake control.

Several previous reports have demonstrated that ectopic expressions of NB-LRR or PRR genes cause induction of downstream defense responses in the absence of the cognate effector or PAMP [14–17]. For example, overexpression of *Pto* displayed broad spectrum resistance to bacterial and fungal pathogens including *Pseudomonas syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria*, and *Cladosporium fulvum* [15]. Consistent with these previous studies, overexpression of *Xa21* confers enhanced resistance and seedling stage and is accompanied by partial induction of defense-related genes. However, *Xa21*-overexpressing plants are still susceptible to an *Xoo* strain lacking *Ax21* activity and to *M. oryzae*. We hypothesize that, in the absence of *Ax21*, the *XA21*-mediated resistance pathway in the Ubi *Myc-XA21* plants is not fully activated.

In contrast to *Pto*-overexpressing tomato plants, which display spontaneous cell death [15], overexpression of *Xa21* enhances resistance at the seedling stage with no observable detrimental effects on plant growth or development. Thus constitutive expression of *Xa21* provides a useful strategy to engineer resistance to *Xoo* during multiple developmental stages.

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Competing interests: The authors have declared that no competing interests exist.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2010.07.008.

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